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The oxygen reaction of the cytochrome *d*-terminated respiratory chain of *Escherichia coli* at sub-zero temperatures

Kinetic resolution by EPR spectroscopy of two high-spin cytochromes

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The oxygen reaction of the fully reduced respiratory chain in membranes from oxygen-limited *Escherichia coli* was studied at sub-zero temperatures using EPR spectroscopy. Laser photolysis of CO-liganded cytochrome oxidase *d* precedes oxidation of at least 2 kinetically separable high-spin cytochromes. At −120 to −100°C, a rhombic signal appears, attributable to cytochrome *d*, followed at above −100°C, by appearance of a second, axial signal near $g=6$, here assigned to cytochrome(s) *b*, and changes in the redox state of iron-sulphur clusters. The data kinetically resolve the 2 high-spin signals attributed to the oxidase complex and suggest schemes for electron flow to oxygen.

Cytochrome oxidase Escherichia coli *Cytochrome* b-d *complex* *Bacterial electron transport* *Low-temperature technique*

1. INTRODUCTION

Escherichia coli possesses 2 terminal cytochrome oxidases (reviews [1–3]). One, cytochrome *o*, has been extensively studied and was the first bacterial oxidase in which an oxygenated intermediate was detected at sub-zero temperatures [4]. The other oxidase, cytochrome *d*, has a higher oxygen affinity and is more resistant to respiratory inhibitors. It has been purified as a 'cytochrome *b*-558–*d* complex' [5,6]. This also contains a so-called 'cytochrome a_1', which probably has a *b*-type haem [7] and resembles haemoprotein *b*-590, a soluble hydroperoxidase [8]. We previously studied the *E. coli* cytochrome *d*-O_2 reaction by the low-temperature triple-trapping method [9]. Photolysis of carbonmonoxycytochrome *d* in the presence of O_2 at −130°C gives an intense absorption band at 650–652 nm with respect to the reduced, CO-bound state [10,11] attributable to an oxygen adduct of reduced cytochrome *d* [10,12]. Here, we present complementary EPR studies that provide kinetic resolution of 2 high-spin EPR signals, both previously attributed to cytochrome *d*.

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2. MATERIALS AND METHODS

2.1. *Organism, growth conditions and membrane preparation*

E. coli K12 (strain A1002) was grown under

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oxygen-limited conditions [13] except that succinate was at 20 mM. Batch cultures (6 l) were sparged with sterile air at 0.6 l air · min^{-1} and slowly stirred to give an O_2 transfer rate of approx. 8.4 mmol · l^{-1} · h^{-1} [14]. Cells were harvested and washed [4] after 19–23 h growth when E_{600} was 0.4–0.8. Washed cell pellets, stored at −20°C, were thawed, washed again with buffer [4], resuspended at about 0.33 g (wet wt cells) · ml^{-1} and used for preparation of particles [15].

2.2. *Low-temperature methods*

Membranes (approx. 30 mg protein per ml of the above buffer, supplemented with 30% (v/v) ethylene glycol [9]) were transferred to a standard EPR tube, reduced with 6 mM Na succinate and bubbled with CO for 15 min at 0°C. The tube was cooled to −23.5°C in dry ice-ethanol for 10 min and supplemented with O_2 by stirring with 5 strokes of a close-fitting coiled stainless-steel wire before rapidly freezing at −78°C. Between 5 and 30% of the CO-liganded oxidase becomes oxidized during oxygenation due to ligand exchange [10,11]. Photolysis was achieved by irradiating the frozen sample, maintained at −140°C in a cryostat, with the beam from a 0.95 mW He-Ne laser (Spectra Physics, Mount View, CA 84042) for 5 min. The laser line (632.8 nm) photolyzes the CO compound of reduced cytochrome *d* (absorption maximum at about 636 nm) but gives negligible photolysis of cytochrome *o* [13]. The sample was then incubated at −140°C for a further 10 min, transferred to the EPR cavity at liquid helium temperature and the EPR spectrum of the sample obtained. The sample was again transferred to the incubation cryostat at the desired temperature, incubated for a further 10 min and transferred to the EPR cavity. Although it would have been desirable to photolyze the CO compound at 77 K where the reaction of the reduced enzyme with CO or O_2 would be negligible (e.g. [16]) this approach is not appropriate for cytochrome *d*. Unlike mammalian cytochrome oxidase, photolysis at temperatures below about −150°C results in rapid recombination of the oxidase with CO even in the presence of O_2 [17]. Spectra were run on a Varian 109 X-Band spectrometer equipped with a Nicolet computer for data accumulation and analysis, and an Air Products liquid He cryostat.

3. RESULTS

Fig.1 shows EPR spectra after incubating membranes at different sub-zero temperatures after photolysis of carbon monoxycytochrome *d*. Immediately after photolysis and before reaction with the oxygen in the frozen suspensions, all redox centres will be reduced and cytochrome oxidase *o* remains bound to CO; in practice, this state is not

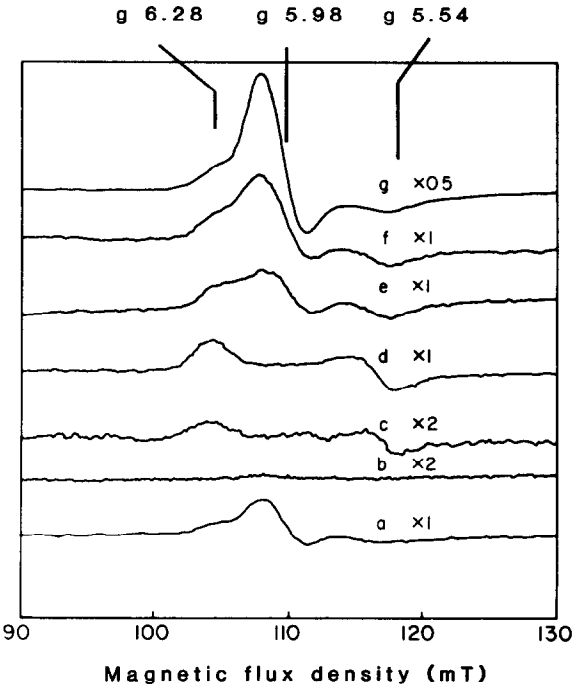

Fig.1. Development of EPR signals in the $g = 6$ region. Membranes in 30% (v/v) ethylene glycol were reduced with succinate, saturated with CO and taken to −23°C where 400 μM O_2 was added before freezing. The spectrum of this form (containing a small quantity of oxidized haem, spectrum a) was recorded as the baseline and subtracted from all subsequent scans. In (b), the sample was then photolyzed with an He-Ne laser at −140°C, incubated at that temperature for 15 min and returned to the measuring temperature of the spectrometer (10 K) and the EPR spectrum obtained to give the difference spectrum shown. The same sample was re-warmed to −120 (c), −100 (d), −80 (e), −60 (f) and −40°C (g), for 10 min in each case and the difference spectrum plotted after each cycle. EPR operating conditions were: microwave power, 2 mW; microwave frequency, 9.168 GHz; modulation amplitude, 2.0 mT; time constant, 0.25 s; scan time, 20 min (2 accumulations).

observed after photolysis at −140°C, oxygen having already reacted with reduced cytochrome *d* giving 'cytochrome *d*-650' [10]. At −140°C, there is no change in the EPR spectrum of signals near $g = 6$. This is most clearly seen in fig.1b, the computed difference between the pre- and post-photolysis states. Between −120 and −100°C, an EPR signal appears, attributed to high-spin haem. The line shape is that of a rhombically distorted component. By −80°C, the rhombic signal has acquired full intensity and appears to remain unchanged at higher temperatures. From −80°C to about −40°C, a large signal develops, characteristic of high-spin haem with axial symmetry. The EPR signal at the end of incubation at −40°C in the $g = 6$ region is very similar in shape and intensity to the signals obtained from fully oxidized *E. coli* membranes at similar concentrations (not shown), suggesting that haem oxidation is nearly complete at this point. This is the first kinetic resolution of the 2 signals previously assigned to cytochrome *d* [18,19].

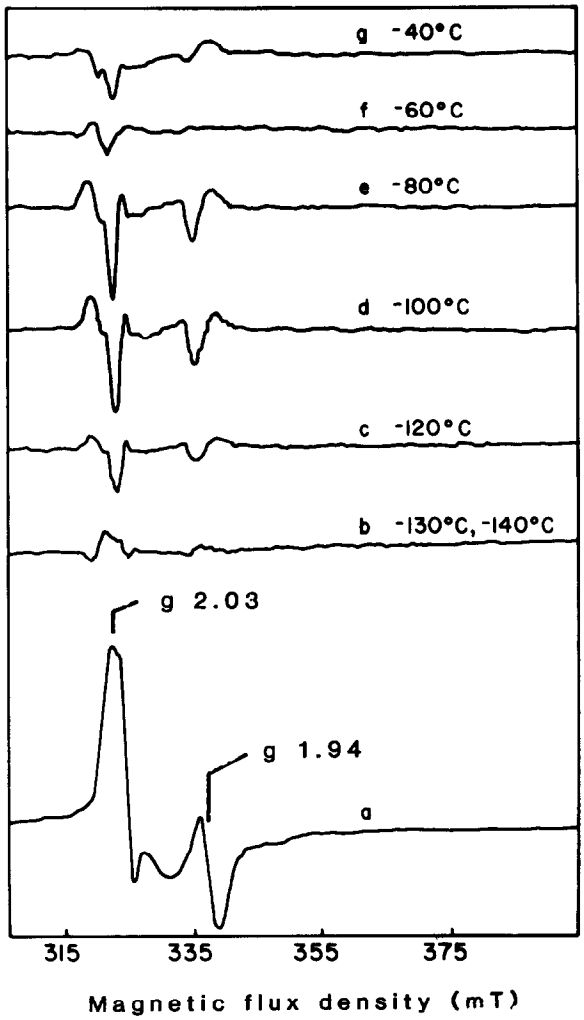

Fig.2. Changes in the EPR spectra in the $g = 2$ region corresponding to data and conditions in fig.1. (a) Absolute spectrum of the succinate-reduced, CO-bound membrane sample. (b–g) Difference spectra obtained by subtracting (a) from the spectrum obtained after incubation of the sample at the specified temperature. EPR conditions as in fig.1 except that modulation amplitude was 1 mT.

Fig.2 shows the corresponding changes occurring near $g = 2$. The major contributors to EPR signals in this region are iron-sulphur proteins. There is some loss of intensity in the EPR signal in the $g = 2$ region during the oxygen reaction between −120 and −80°C, i.e. there is some oxidation of the centres. However, this loss of intensity is reversed (reduction) and the signals almost regain their initial intensity (as in the fully reduced membrane) at −60 to −40°C presumably due to electron transfer into the FeS centres from succinate.

4. DISCUSSION

The EPR results presented here complement previous optical studies (fig.3). Since the rhombic high-spin haem signal appears at very low (< −100°C) temperatures where the only optical changes seen are in cytochrome *d*, and since cytochrome *d* is known to be the oxygen binding site [1,2,10], we tentatively assign the rhombic signal to cytochrome *d*. In *Azotobacter vinelandii* membranes, similar signals (axial, $g = 5.94$; rhombic, $g = 6.24$, $g = 5.51$) were attributed to 2 species of cytochrome *d* [18,19]. CO and cyanide, however, affected predominantly the rhombic component and gave optical changes in the 648 nm band. Since such ligands bind to the O_2-binding haem in other cytochrome oxidases, the results lend support to the present assignment of the rhombic signal to cytochrome *d*. The axial signal (fig.1) probably arises from *b*-type cytochromes, but we cannot yet assign it to either (or both of) cytochrome *b*-556 or the oxidase-associated *b*-558. Previous optical studies indicate that the CO com-

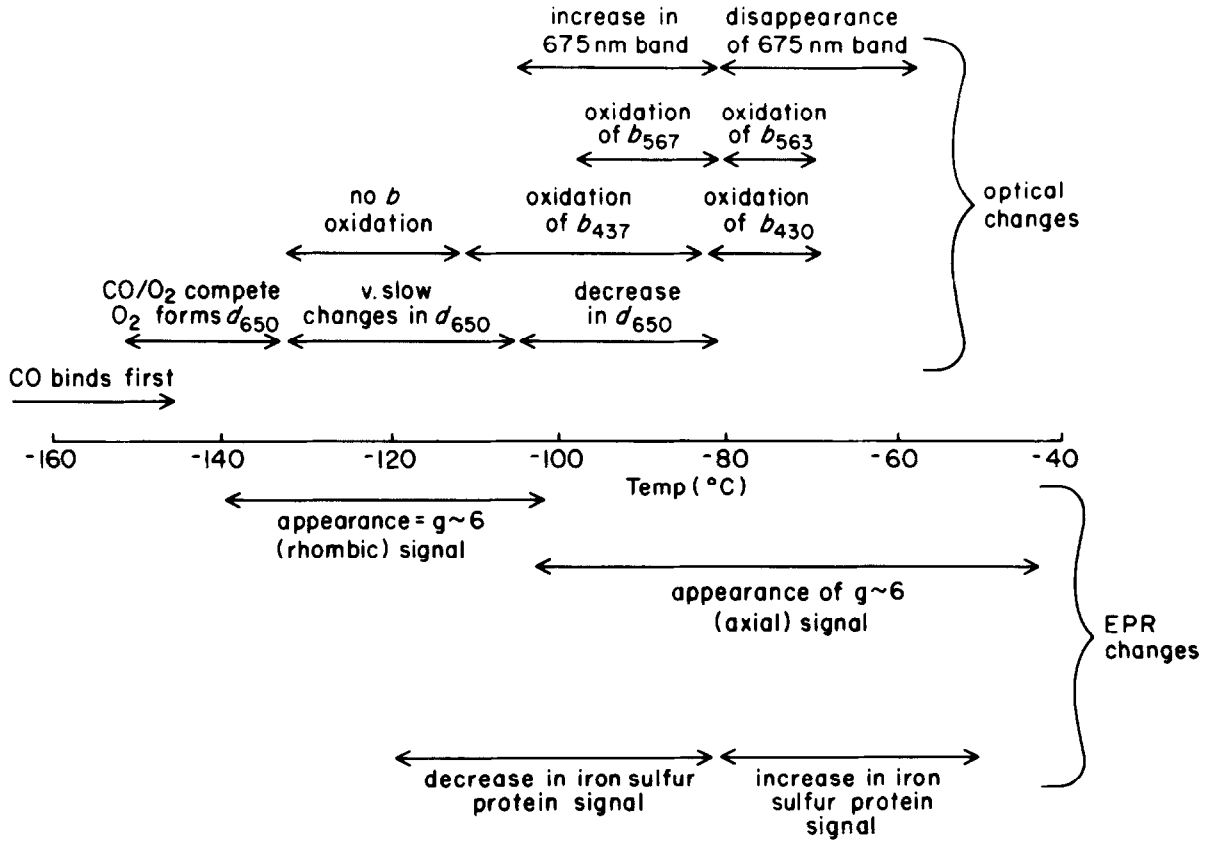

Fig.3. Summary of optical and EPR changes occurring when reduced oxygen-limited *E. coli* membranes react with O_2 at low temperature. The optical changes (above the temperature scale) are those reported in [10,11,17].

pounds of other haemoproteins (e.g. hydroperoxidases) are not dissociated under these conditions and are thus effectively excluded from the EPR difference spectra.

Integration of the high-spin EPR signals at −40°C in fig.1 by the method of Aasa and Vanngard [20] suggests that the rhombic and axial species are in a 1:3 ratio. While supporting our tentative identification of these species, the significance of the stoicheiometry is uncertain. Oxygen must bind to cytochrome *d* in the reduced *bd* complex, and may accept electrons from one or more *d*-type haems and/or from cytochrome *b*. However, below −100°C only the rhombic haem is oxidized and if this occurred via one-electron transfer to O_2, the complex produced (Fe^{3+} O_2^-) would be expected to be EPR-silent. Possible explanations include the following:

(i) The O_2^- produced may interact with another group to form a charge transfer complex (the 650 nm band?) and a magnetically isolated haem. Such a situation may occur in an intermediate of the beef heart oxidase-oxygen reactions [16]. Transfer of a second electron to the superoxide could account for appearance of the 675 nm band (a peroxy form?) and cytochrome *b* oxidation, reflected in the intensity increase of the axial EPR signal.

(ii) The level of reduction of oxygen under conditions where the rhombic $g = 6$ EPR signal alone is observed may be peroxide. If, concomitant with electron transfer from cytochrome *d* to oxygen, another group also transfers an electron to the oxygen, it would be reduced to diamagnetic peroxide, leading to observable EPR signals from cytochrome *d*. This second group could be EPR

undetectable or perhaps a second haem *d*.

Alternatively, the rhombic EPR signal arises not from cytochrome *d* but from some other cytochrome component of the complex. In this interpretation, reduced cytochrome *d* binds oxygen to produce an EPR-silent oxidized haem *d*-superoxide complex and a remote, magnetically isolated oxidized cytochrome component accounting for the rhombic high-spin EPR signal. A choice between these and other explanations can be made only on the basis of further study, especially of the purified enzyme.

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